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(54) Title: SOLUBLE GLUCANS

(57) Abstract

A new class of soluble glucans is described as well as the process for making the same. According to a preferred embodiment, the glucan is a glucan sulfate derived from the yeast *Saccharomyces cerevisiae*. The glucan sulfates are useful for prophylactic and therapeutic applications against infections induced bacteria, viral, fungal and parasitic agents. Additionally, they may be administered to stimulate hematopoietic bone marrow activity and to stimulate macrophage cell phagocytic activity *in vivo*. The glucan sulfates are also useful agents against neoplastic diseases.

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SOLUBLE GLUCANS

1. FIELD OF THE INVENTION

This invention relates to a new class of soluble glucans and, more particularly, to soluble glucans in which the polyglucopyranose chains are solubilized by the addition of a polar charged chemical group as well as to processes for preparing these new soluble glucans from various microorganisms, such as *Saccharomyces cerevisiae*, through the addition of a polar charged group by reaction with a non-phosphorous containing hydrolytic acid. The new soluble glucans of the invention are non-toxic and exert pronounced immunobiological responses when administered *in vivo*, the most notable activities being immunostimulation of macrophage activity and stimulation of hematopoietic bone marrow activity. Additionally, these soluble glucans exhibit significant effects *in vivo* against malignant neoplasms including melanomas and sarcomas.

2. BACKGROUND OF THE INVENTION

The term "glucan" refers generically to a variety of naturally occurring homopolysaccharides or polyglucoses, including polymers such as cellulose, amylose, glycogen, laminarians, starch, etc. Glucan encompasses branched and unbranched chains of glucose units linked by 1-3, 1-4, and 1-6 glucosidic bonds that may be of either the alpha or beta type.

As defined herein, "particulate glucan" designates a water-insoluble particulate (about 1-3 μ) polyglucose such as that derived from the cell wall of the yeast *Saccharomyces cerevisiae*. Particulate glucan is macromolecular and comprises a closed chain of glucopyranose units united by a series of β-1-3

glucosidic linkages. (Hassid et al., 1941, J. Amer. Chem. Soc. 63: 295-298; Di Luzio et al., 1979, Int'l J. Cancer 24: 773-779). X-ray diffraction studies have demonstrated that particulate glucans exist in the form of a triple-stranded helix. (Sarko et al., 1983, Biochem. Soc. Trans. 11: 139-142).

Also as defined herein, the term "glucan phosphate" or "soluble phosphorylated glucan" refers to the class of glucans solubilized by the addition of charged phosphate groups through reaction with phosphoric acid. These are the same or substantially similar to those substances as described in U.S. Patents Nos. 4,739,046; 4,761,402; 4,818,752 and 4,833,131.

2.1 IMMUNOBIOLOGICAL ACTIVITIES OF PARTICULATE GLUCANS

Particulate glucan is a potent activator of the macrophage/monocyte cell series, complement, as well as T and B cell lymphocytes. Thus, particulate glucan has profound effects on both the reticuloendothelial and immune systems.

Previous studies have demonstrated that *in vivo* administration of particulate glucan to a variety of experimental animals induces a number of profound immunobiological responses, including the following: (1) enhanced proliferation of monocytes and macrophages (Deimann and Fahimi, 1979, J. Exper. Med. 149: 883-897; Ashworth et al., 1963, Exper. Molec. Pathol., Supp. 1: 83-103); (2) enhanced macrophage phagocytic function (Riggi and Di Luzio, 1961, Am. J. Physiol. 200: 297-300); (3) enhanced macrophage secretory activity (Berlin et al., 1981, in Heterogeneity of Mononuclear Phagocytes, Forster and Landy, eds., Academic Press, New York, pp. 243-252); (4) increased macrophage size (Patchen and Lotzova,

1980, Exper. Hematol. 8: 409-422); (5) enhanced macrophage adherence and chemotactic activity (Niskanen *et al.*, 1978, Cancer Res. 38: 1406-1409); and (6) enhanced complement activation (Glovsky *et al.*, 1983, J. Reticuloendothel. Soc. 33: 401-413). Increased cytolytic activity against tumor cells has been demonstrated in macrophages from animals and man treated with particulate glucan in both *in vivo* (Mansell and Di Luzio, 1976, in "The Macrophage in Neoplasia", Fink, ed., Academic Press, New York, pp. 227-243) and *in vitro* studies (Chirigos *et al.*, 1978, Cancer Res. 38: 1085-1091).

Stimulation of the reticuloendothelial system by *in vivo* administration of particulate glucan leads to inhibition of allogenic or xenogeneic bone marrow graft acceptance in lethally irradiated animals. (See, *e.g.* Wooles and Di Luzio, 1964, Proc. Soc. Exper. Biol. Med. 115: 756-759). This finding denotes that glucan will induce host defense mechanisms even against normal cells if they are genetically different from the host.

In addition to effects on reticuloendothelial and immune responses, *in vivo* administration of particulate glucan has been demonstrated to enhance hemopoietic activity including granulopoiesis, monocytopoiesis and erythropoiesis leading to greater recovery from a lethal dose of whole body irradiation (Patchen, 1983, Surv. Immunol. Res. 2: 237-242).

A number of studies have indicated that *in vivo* administration of particulate glucan significantly modifies host resistance to a wide variety of infectious diseases induced by bacterial, fungal, viral and parasitic organisms. In particular, enhanced host resistance to infection has been shown

when animals are challenged by microorganisms such as <u>Eshericheria coli</u>, <u>Staphylococcus aureus</u>, <u>Francisella tularensis</u>, <u>Mycobacterium leprae</u>, <u>Streptococcus pneumoniae</u>, <u>Candida albicans</u>, <u>Sporotrichum schenckii</u>, as well as viruses such as Venezuelan equine encephalomyelitis virus, Rift Valley fever virus, murine hepatitis virus, frog virus III, Herpes simplex I and II, and parasites such as <u>Leishmania donovani</u> (see review by Di Luzio, 1983, Trends in Pharmacol. Sci. <u>4</u>: 344-347 and references cited therein).

Extensive studies have indicated that particulate glucan has potent anti-tumor activity. For example, particulate glucan has been shown to inhibit tumor growth and prolong survival in four syngeneic murine tumor models including adenocarcinoma BW 10232, anaplastic carcinoma 15091A, melanoma B16, and spontaneous lymphocytic leukemia BW5147 (Di Luzio <u>et al</u>, 1979, <u>in</u> Advances in Experimental Medicine and Biology, Vol. 121A: 269-290).

To evaluate the cellular basis of the anti-tumor activity of particulate glucan, the anti-tumor cytotoxic activity of peritoneal macrophages, derived from control and particulate glucan-treated mice, was studied (Mansell and Di Luzio, 1976, <u>in</u> The Machrophage in Neoplasia, Fink, ed. Academic Press, New York, pp. 227-243). These studies indicated that peritoneal macrophages from glucan-treated mice produced a significant cytotoxic response compared to normal macrophages. This observation has been confirmed (See, <u>e.g.</u>, (Berlin <u>et al</u>. 1981, in Heterogeneity of Mononuclear Phagocytes, Forster and Landy, eds., Academic Press, New York, pp. 243-252) and Chirigos <u>et al</u>., 1978, Cancer Res. <u>38</u>: 1085-1091).

Additionally *in vitro* studies using normal and tumor cells incubated with particulate glucan have demonstrated that glucan exerts a direct cytostatic effect on sarcoma and melanoma cells and a proliferative effect on normal spleen and bone marrow cells (Williams *et al*., 1985, Hepatology, 5: 198-206). These studies indicate that the glucan, when administered therapeutically, will (1) significantly inhibit hepatic metastases; (2) inhibit the growth of the primary tumor; and (3) enhance survival, possibly by increased Kupffer cell tumoricidal activity as well as by a direct cytostatic effect of such glucan on sarcoma cells.

Notwithstanding these biological properties, the adverse side effects of particulate glucans have made these compounds all but useless in clinical medicine.

2.2 ADVERSE SIDE EFFECTS OF PARTICULATE GLUCANS

The adverse side effects of particulate glucans have made these compounds all but useless in clinical medicine. When particulate glucan is administered *in vivo* to animals, a number of severe side effects have become apparent, the most notable being:

(1) formation of granuloma (sarcoidosis);
(2) development of hepatosplenomegaly;
(3) increased susceptibility to gram-negative infections and endotoxins;
(4) activation of complement (anaphylyatoxin);
(5) development of pulmonary granulomatous vasculitis;
(6) development of hypotension following intravenous administration; and

(7) development of microembolism when administered at high concentrations.

Additionally, there is a relatively high degree of acute toxicity observed when particulate glucan is administered in vivo. For example, following a single intravenous injection of an aqueous suspension of particulate glucan, 20% and 100% mortality were observed in mice receiving glucan at 250 and 500 mg/kg body weight respectively.

Moreover, due to the particulate nature of the glucan preparation (1-3 μ), it is difficult to administer via an intravenous route. By way of illustration, one patient receiving particulate glucan required constant supervision during intravenous (IV) administration, continuous shaking of the IV drip bottle being essential to maintain the particulate glucan in suspension to avoid formation of emboli in the patient.

Although slightly soluble neutral glucans are commercially available, these preparations are not suitable for intravenous administration because the aqueous solutions have very high viscosity and, more importantly, because their use when administered to experimental animals has inevitably been accompanied by considerable toxicity.

Lentinan, a high molecular weight and poorly soluble β-1,3 and β-1,6 glucan obtained from Lentinus edodes, has been studied following intravenous administration to dogs. A variety of adverse clinical effects were observed following administration of lentinan (Ajinomoto Co. Inc., Tokyo, Japan) at doses of 2.0, 8.0 and 30 mg/kg/day for 5 weeks. Adverse effects included vomiting, erythema, discoloration of the sclera, and facial swelling. Circulatory collapse, unsteady gait, altered behavioral patterns,

excessive salivation were also seen in individual beagles. At autopsy, congestion of the gastrointestinal mucosa was observed in animals treated with 2.0 or 8.0 mg/kg/day. Morphological changes of liver indicated intracytoplasmic material, possibly lentinan, accumulating in liver cells. One animal showed circulatory collapse upon the first injection at 8.0 mg/kg. While he did recover, the animal experienced repeated vomiting episodes with presence of blood indicating hemorrhaging of the gastrointestinal tract. Another animal appeared to show a marked allergic response, as demonstrated by erythema and subcutaneous swelling (edema) of the face. Autopsy findings demonstrated extensive edema of subcutaneous tissue, and congestion of the gastrointestinal tract with hemorrhaging. Macrophage cells showed accumulation of material, possibly lentinan. (Chesterman et al.,1981, Toxicol. Lett. 9: 87-90)

Additional toxicity studies were performed in which a variety of doses of lentinan ranging from 0.1 to 1.0 mg/kg/day were given intravenously to rats for 9 weeks. Toxicity was manifested by the development of cutaneous lesions and discoloration of the ears suggesting thromboembolic events. (Cozens et al., 1981, Toxicol. Lett. 9: 55-64).

2.3 UNSUCCESSFUL ATTEMPTS TO SOLUBILIZE PARTICULATE GLUCANS

In view of these disadvantages of particulate β-1,3 glucans for *in vivo* administration, extensive studies were undertaken to develop a soluble β-1,3 polyglucose which might be non toxic, induce no significant pathology, and yet retain significant immunobiological activity.

A low molecular weight non-phosphorylated soluble glucan preparation prepared by formic acid hydrolysis of particulate glucan has been shown to have anti-tumor and anti-staphylococcal activity (Di Luzio et al., 1979, Internat'l J. Cancer 24: 773-779). Unfortunately, the low yield and diversity of fractions obtained by this method made this preparation non-useful for prophylactic and therapeutic applications. (see Di Luzio, 1983, Trends in Pharmacological Sciences 4: 344-347).

Similarly, attempts to solubilize particulate glucan by the addition of dimethylsulfoxide (DMSO) a "molecular relaxant" were also unsuccessful. It was thought the DMSO would "relax" the triple helical configuration of the glucan molecule. Indeed, particulate glucan dissolves in the presence of DMSO. All attempts to isolate a soluble glucan from the DMSO solution, however, resulted in failure. Upon dilution of the DMSO-glucan solution with various aqueous media such as glucose or saline solutions, the particulate glucan was reformed. Following dilution of the DMSO-soluble glucan solution with saline, all animals receiving injections of these solutions died immediately upon injection due to high concentration of DMSO or the reformation of the particulate glucan. Upon precipitation of the glucan in DMSO solution by the addition of ethanol (100%), the precipitate was collected and lyophilized. When this lyophilized glucan was added to water, the particulate glucan reformed.

Attempts to convert the neutral glucan preparation of particulate glucan to a polar-charged preparation by the addition of phosphate or sulfate groups as well as by acetylation were also unsuccessful. Each of these procedures was conducted

following the solubilization of particulate glucan by DMSO and in each instance the particulate glucan was reformed.

2.4 GLUCAN PHOSPHATE

A neutral preparation of particulate glucan was successfully converted into a stable solubilized form termed "soluble phosphorylated glucan" (hereinafter termed "glucan phosphate") through phosphoric acid hydrolysis using the method described briefly below. This soluble phosphorylated glucan is non-toxic, non-immunogenic, and substantially non-pyrogenic (see U.S. Patent Nos. 4,739,046; 4,761,402; 4,818,752 and 4,833,131).

According to this method, glucan phosphate was prepared as follows: particulate glucan was suspended in DSMO. Urea was added, the mixture heated and maintained at 50-150°C while phosphoric acid was slowly added. The product was isolated and the DMSO, urea, glucose, and any unreacted phosphoric acid was removed.

The solubility of glucan phosphate as obtained from S. cerevisiae prepared according to the above method is greater than about 50 mg/ml in water. Its elemental composition is illustrated in Table 1. (see U.S. Patent Nos. 4,739,046; 4,761,402; 4,818,752 and 4,833,131.) The repeat unit empirical formula of glucan phosphate using this preparation is:

$C_{40}H_{87}PO_{37}$.

TABLE 1

ELEMENTAL COMPOSITION OF GLUCAN PHOSPHATE

Element or Component	Mole %
Carbon	34.66
Hydrogen	6.29
Oxygen	42.83
Nitroge	0.64
Sulfur	0.11
Phosphorus	2.23
Water of Hydration	11.78

Previous studies have shown that glucan phosphate exhibits significant immunobiological activity when administered in vivo and in vitro. Therapeutic and prophylactic applications of glucan phosphate have been demonstrated to increase the host resistance of both normal and immunosuppressed animals to a variety of infectious diseases induced by bacterial, fungal, viral and parasitic organisms. (see U.S. Patent No. 4,761,402). Glucan phosphate was also found to be useful in the treatment of neoplastic diseases, either alone or in combination with an anti-tumor agent. Studies have indicated that glucan phosphate stimulates macrophages to produce potent anti-tumor cytotoxins (see U.S. Patent No. 4,818,752).

In addition, studies have demonstrated that glucan phosphate is useful in preventing the development of leukopenia induced by the administration of anti-cancer agents. (see U.S. Patent No. 4,739,046). Due to its ability to

stimulate macrophage secretory and phagocytic activity, glucan phosphate has also been found effective in promoting wound healing. (see U.S. Patent No. 4,833,131).

3. <u>SUMMARY OF THE INVENTION</u>

During an investigation of the methods whereby charged groups might be added to the structure of particulate glucan so that the polymer could be solubilized in aqueous media, it was found that when non-phosphorous containing hydrolytic acids were reacted with particulate glucan, along with a highly polar solvent (such as DMSO) and a strong chaotropic agent (such as urea), the resulting glucan acquired the polar charged group of such acids in varying degrees.

Based on this discovery, the present invention provides a new class of soluble glucans (a) in which the polyglucopyranose chains have acquired a charged group from a non-phosphorous containing hydrolytic acid; (b) which are non-toxic; and (c) which surprisingly are capable of exerting pronounced immunobiological responses when administered *in vivo* in animals and humans. These new soluble glucans immunostimulate macrophage activity with resulting activation of other immunoactive cells in the reticuloendothelial and immune systems. Additionally these soluble glucans enhance hematopoietic bone marrow activity. These soluble glucans exhibit cytostatic effects against malignant neoplasms including melanomas and sarcomas *in vivo*.

According to one embodiment, the invention provides a process for producing a soluble glucan by solubilizing a particulate glucan, preferably prepared

from Saccharomyces cerevisiae although other microbial sources may be used in a highly polar solvent which contains a strong chaotropic agent, reacting the resultant glucan with:

(a) sulfuric acid or

(b) a mixture of sulfuric acid and a highly polar solvent and recovering the resultant soluble glucan from the reaction mixture.

According to another embodiment, the invention provides a process for producing a soluble glucan by solubilizing a particulate glucan, preferably from Saccharomyces cerevisiae, although other microbial sources may be used, in a highly polar solvent which contains a strong chaotropic agent and reacting the resultant glucan with:

(a) glacial acetic acid or

(b) a mixture of glacial acetic acid and a highly polar solvent and recovering the resultant soluble glucan from the reaction mixture.

Further, the invention provides methods for therapeutic and prophylactic treatment of infections induced by bacteria, fungi, viruses and parasitic organisms by administration of soluble glucans or pharmaceutical compositions comprising soluble glucans in combination with a physiologically acceptable carrier.

Additionally, the present invention provides methods for treatment of malignant neoplastic disease in animals and humans, including but not limited to melanoma and reticulum cell sarcoma, by administering to an animal or a human a therapeutically effective amount of a soluble glucan alone or in combination with an anti-cancer agent.

Furthermore, the invention provides methods for stimulating animal and human macrophage phagocytic

activity by the administration of a soluble glucan. Methods are also provided for the stimulation of hematopoietic bone marrow activity by the administration of a soluble glucan.

The immunobiological properties of the soluble glucans of the invention include (1) the ability to significantly modify viral infections; (2) the ability to modify infections induced by bacterial, fungal and other parasitic microorganisms; (3) the ability to increase survival in animals and humans with malignant neoplasms; (4) the ability to enhance hematopoiesis as assessed by bone marrow proliferation; and (5) the ability to increase macrophage phagocytic activity.

Because of these unique properties, the soluble glucans are particularly useful for prophylactic and therapeutic applications against a variety of diseases induced by bacteria, viruses, fungi and parasitic organisms, as well as a number of neoplastic conditions. The soluble glucan compositions may advantageously be used with a physiologically acceptable pharmaceutical carrier, either alone or in combination with other bioactive or pharmacological agents and therapeutic modalities.

4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more fully understood by reference to the following detailed description of the invention, examples of specific embodiments of the invention, and the appended figures in which:

FIG. 1(A-C) illustrates the high performance size exclusion chromatograms for glucan sulfate A, glucan sulfate B and glucan phosphate using an on-line multi-angle laser light scattering photometer. FIG.

1A is the chromatogram for glucan sulfate A. Two polymer peaks with molecular weight averages of 781,768.8 daltons and 5,710.4 daltons were resolved. FIG. 1B is the chromatogram for glucan sulfate B. Two polymer peaks with MW averages of 1,246,441 daltons and 14,513 daltons were resolved. FIG. 1C, included for comparison, is the chromatogram for glucan phosphate. Two polymer peaks with MW averages of 378,772 daltons and 95,963 daltons were resolved.

FIG. 2(A-B) illustrates helical coil transition analyses. Dextran (70kD) served as the linear control and Congo red in sodium hydroxide served as the negative control. FIG 2A is the helical coil analysis for glucan sulfate A. It shows a shift in absorption maxima which is not consistent with that observed for compounds with a triple helical conformation. FIG. 2B is the helical coil transition analysis of glucan sulfate B. It shows a shift in absorption maxima which is characteristic of compounds exhibiting a triple helical conformation. Included for comparison in both FIG. 2(A and B), is the helical coil transition analysis for glucan phosphate which shows the same shift characteristic of triple helical compounds. It served as the triple helical control for FIG. 2A and FIG. 2B.

FIG. 3(A-D) includes representations of the nuclear magnetic resonance spectrum for glucan phosphate, laminarin, glucan sulfate B and glucan sulfate A. FIG. 3A, included for comparison, is the ^{13}C-NMR representation for glucan phosphate at a concentration of 50 mg/ml. FIG. 3B is the ^{13}C-NMR spectrum of a commercially available preparation of laminarin standard (Lot 95284 - K & K Laboratories, Inc., Plainview, NY) at a concentration of about 30 mg/ml. FIG. 3C is the ^{13}C-NMR representation for

glucan sulfate B at a concentration of 50 mg/ml. FIG. 3D is the ^{13}C-NMR representation for glucan sulfate A at a concentration of 50 mg/ml.

FIG. 4 is a graph illustrating the effect of administration of a glucan sulfate on survival of mice with subsequent experimentally induced Candida albicans infection.

FIG. 5 is a graph illustrating the effect of prior treatment with a glucan sulfate on survival of mice with subsequent experimentally induced viral hepatitis.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1 PROCESSES FOR PREPARATION OF SOLUBLE GLUCANS

Aqueous soluble glucans are prepared by a process comprising reacting a particulate glucan with a non-phosphorous containing hydrolytic acid containing a polar charged group. Examples of useful, non-phosphorous containing hydrolytic acids, include nitric acid, sulfuric acid, acetic acid, etc. The polar charged group, examples of which include nitrate, sulfate and acetate groups, is added onto the structure of the glucan in the presence of a highly polar solvent and a strong chaotropic agent.

According to one embodiment of the present invention, soluble glucan sulfate is prepared by one of two processes, both which result in a unique class of products different from any other glucans previously described. Soluble glucan sulfate prepared by the first method is herein designated as glucan sulfate A, while that prepared by the second method is herein designated glucan sulfate B.

According to a preferred mode, i.e. the first method, of this embodiment of the present

invention, soluble glucan sulfate is prepared as follows: particulate glucan, derived from <u>Saccharomyces</u> <u>cerevisiae</u>, is suspended in a solution of a strong chaotropic agent in an aprotic solvent such as dimethylsulfoxide (DMSO) with constant stirring. The strong chaotropic agent "relaxes" hydrogen bonding along the polyglucose chain, thus unfolding the molecule. It is preferred to use a fairly high concentration of a strong chaotropic agent such as urea ranging from about 4-12 M to prevent reformation of hydrogen bonds. The mixture is then heated and maintained at about 50-150°C and sulfuric acid is added. A precipitate is apparent after about 1.5 hours. After reaction for about 6 hours at about 100°C, the yield is approximately 37.5%. The bioactive glucan sulfate product is isolated from the reaction mixture by resuspending or dissolving the precipitate by the addition of a sufficient amount of water and filtering the mixture through a coarse sintered filter. The glucan sulfate is further isolated from the solution as described below.

According to a more preferred mode of this embodiment of the present invention, i.e. the second method, soluble glucan sulfate is prepared by a preparation procedure which is substantially similar to the first method, with the following exception: a mixture of sulfuric acid (1/4 the amount employed in the first method) and DMSO is added to solubilize the particulate glucan, as opposed to sulfuric acid alone. The DMSO is thought to buffer the hydrolytic effect of sulfuric acid, thus decreasing the degree of hydrolysis of the glucan polymers and increasing the percentage of conversion to the soluble material. This increases the yield from 37.5% using the first method to about 98% using the second method. Although

applicant does not intend to be limited by the following explanation, it is presently thought that hydrolysis of the polymers and/or addition of polar charged groups to the polymers may contribute to the degree of solubilization of the particulate glucan. However, it has not been determined whether hydrolysis is required for solubilization of the particulate material.

The bioactive glucan sulfate product is isolated from the reaction mixture as follows: the mixture is cooled to stop the sulfation reaction and diluted with a volume of water sufficient to resuspend any precipitate. The resulting solution is filtered through a series of filters (e.g., 1-3μ, 0.6μ, and 0.45μ filters) to remove any remaining precipitate and any colloidal particles. The solution is then molecularly sieved to remove all components of less than about 10,000 daltons molecular weight (MW). Thus, DMSO, urea, glucose and any unreacted sulfuric acid are removed from the solution. Molecular sieving may be accomplished by any method that removes these low (i.e., less than about 10,000 daltons) MW components. In one illustrative example, the solution is sieved using a Millipore dialyzer/concentrator with a 10,000 daltons MW membrane filter and a large volume of dialyzing solution. Following molecular sieving, the resulting solution is concentrated, shell frozen and lyophilized to yield the final product.

A similar method is used for the preparation of glucan acetate. According to this embodiment of the invention, soluble glucan acetate is prepared as follows: particulate glucan, preferably derived from Saccharomyces cerevisiae, is suspended in a solution of a strong chaotropic agent, such as urea, in an aprotic solvent such as DMSO with constant stirring.

It is preferred to use about 4-12 M of the chaotropic agent. The mixture is heated to about 50-100°C and glacial acetic acid is added. No precipitate forms and the reaction is allowed to proceed for about 6 hours at about 100°C. The reaction is stopped by lowering the temperature, the mixture is diluted with deionized water and filtered to remove any remaining particulate or colloidal material.

According to another mode of this embodiment of the present invention, particulate glucan is suspended in DMSO containing urea and the mixture is heated and maintained at about 50-150°C. A DMSO/acetic acid mixture is then added. This procedure does not result in the formation of a precipitate and the reaction is allowed to proceed for about 6 hours at 100°C. The mixture is cooled to stop the reaction, diluted with deionized water, and filtered to remove any remaining particulate or colloidal material. The solution is then dialyzed and concentrated using, for example, tangential flow ultrafiltration. The resulting material is shell frozen and lyophilized.

The particulate glucan used in the processes for preparing the soluble glucans according to the present invention may be isolated from the cell wall of S. cerevisiae by known methods (see e.g., Di Luzio et al., 1979, Internat'l J. Cancer 24: 773-779; Hassid et al., 1941, J. Amer. Chem. Soc. 63: 295-298 incorporated herein by reference). Briefly, in practice the particulate glucan is prepared as follows: dry yeast is digested in aqueous sodium hydroxide solution and heated to about 100°C and maintained for about 4 hours, then cooled overnight. The supernatant is decanted and the procedure is repeated three times. The residue is acidified using

hydrochloric acid and maintained at 100°C for about 4 hours, then cooled overnight. The supernatant is decanted and the acid digestion is repeated twice. The residue is then washed repeatedly with distilled water and extracted with ethanol for at least 24 hours. The reddish-brown supernatant is then aspirated and discarded. The ethanol extraction is repeated until the supernatant is essentially colorless. The ethanol is removed by repeatedly washing the residue with distilled water. The particulate glucan is collected by centrifugation or filtration.

According to alternative embodiments of the present invention, soluble glucans can be prepared from neutral polyglucose or polyglucose-protein products derived from a variety of other microbial sources. A non-exhaustive list of such sources is presented in Table 2.

TABLE 2

EXAMPLES OF SOURCES OF GLUCAN WHICH CAN BE EMPLOYED FOR THE PREPARATION OF SOLUBLE GLUCANS

Alcaligenes faecalis
Auricularia auricula-judae
Auricularia polytricha
Candida utilis
Cladosporium fulvum
Claviceps purpurea
Cochiliobolus sativus
Coriolus versicolor
Corlinellus shiitake
Corticium vagum
Grifola umbellata
Lentinus edodes
Pichia fermentans
Poria cocos
Saccharomyces cerevisiae
Sclerotium coffeicolum
Sclerotium delphnii
Sclerotium glucanicum
Sclerotium rolfii
Shizophyllum commune
Streptococcus salvarius
Stereum sanguinolentum
Wingea robertsii

5.2 CHARACTERIZATION OF SOLUBLE GLUCANS

The solubility of soluble glucan as obtained from S. cerevisiae prepared according to the first method of the present invention using sulfuric acid as

the hydrolytic acid (herein designated glucan sulfate A) has been determined to be greater then 400 mg/ml in water. The solubility of soluble glucan prepared according to the second method of the present invention using sulfuric acid as the hydrolytic acid (herein designated glucan sulfate B) is 50 mg/ml in water. Additionally, glucan sulfate A hydrates faster than either glucan sulfate B or glucan phosphate.

5.2.1 ELEMENTAL COMPOSITION

The elemental composition of both the glucan sulfate A and glucan sulfate B preparations, as determined by Gailbraith Laboratories (Knoxville, TN) is illustrated in Table 3. The nitrogen in glucan sulfate B is thought to originate from the urea used as a chaotropic agent in the solubilization of glucan sulfate B. The data presented in Table 3 permits the following repeating unit empirical formulas to be derived:

Glucan Sulfate A $(C_6H_{10}O_5)_7 \cdot (HSO_4)_5$

Glucan Sulfate B $(C_6H_{10}O_5)_8 \cdot 3SO_3NH_4^+ \cdot 4H_2O$

Thus, there is an average of one sulfate group for every two glucose subunits in glucan sulfate A, and an average of one sulfate group for every three glucose subunits in glucan sulfate B. Although applicant does not intend to be limited to any mechanism or explanation, the solubility of glucan sulfate B suggests that it exists in the form of an ammonium sulfate salt.

TABLE 3

ELEMENTAL COMPOSITION OF GLUCAN SULFATE A AND GLUCAN SULFATE B*

Element	Glucan Sulfate A	Glucan Sulfate B
Carbon	26.3	34.06
Hydrogen	5.18	6.15
Oxygen	59.34	50.30
Sulfur	8.30	5.69
Nitrogen	-	3.23

* Value expressed as mole %.

5.2.1.1 HIGH PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

Aqueous high performance size exclusion chromatography using Ultrahydrogel columns (Waters Chromatography Division / Millipore Corporation, Milford, MA), a Wyatt multi-angle laser light scattering photometer (Wyatt Technology, Santa Barbara, CA) and an on-line differential viscometer (Viscotek, Porter, TX) was employed to determine the average MW, peak MW, number average, polydispersity number and intrinsic viscosity of glucan sulfate A and B. The data are presented in Table 4. For comparison, the data for glucan phosphate is shown as well. The number average is an index of the amount of low molecular weight polymers present. The polydispersity number reflects polymer homogeneity (i.e., the lower the number, the more homogenous the polymers are with regard to molecular weight).

TABLE 4

MOLECULAR WEIGHT AND POLYMER DISTRIBUTION DATA

	Glucan Sulfate A	Glucan Sulfate B	Glucan Phosphate
	Peak 1		
Number Average	320,209.6	510,445	193,541
Weight Average (D)	781,768.6	1,246,441	378,772
Peak MW (Mp)	240,000	1,590,000	813,000
Polydispersity	2.44	2.44	1.96
	Peak 2		
Number Average	5,470.7	13,487.6	84,178.2
Weight Average (D)	5,710.4	14,487.6	95,936.0
Peak MW (Mp)	5,700	13,100	77,300
Polydispersity	1.04	1.08	1.14
Intrinsic Vixcosity dl/g	.081	1.08	.317

The data from the high performance size exclusion chromatography indicate that there are two polymer or molecular weight peaks in both glucan sulfate A and glucan sulfate B (see FIG. 1A and FIG. 1B). The chromatogram of glucan phosphate (FIG. 1C), shown here for comparison, also indicates two polymer peaks.

Cumulative MW distributions of glucan sulfate A and glucan sulfate B are shown in Table 5. The cumulative MW distribution for glucan phosphate is shown for comparison.

TABLE 5

CUMULATIVE MOLECULAR WEIGHT DISTRIBUTIONS

Cumulative Molecular Weight Distribution (daltons)	Glucan Sulfate A	Glucan Sulfate B	Glucan Phosphate
	Expressed as cumulative area %		
10,000-100,000	97.30	99.74	82.91
100,000-500,000	2.55	0.07	14.69
500,000-1,000,000	0.61	0.06	1.71
>1,000,000	<0.08	<0.13	0.69

5.2.1.2 ULTRAVIOLET-VISIBLE SPECTROPHOTOMETRY

Conformational structure was assessed using the technique of Ogawa and co-workers (Ogawa and Hatana, 1978, Carbohyd. Res. 67: 527-535; Ogawa and Tsurugi, 1973, Carbohyd. Res. 29: 397 - 403). This technique determines the absorption maxima of polymer solutions complexed with Congo Red in the presence of various concentrations of hydroxide ion. The presence of a triple-helical compound, for example, would be indicated by a shift in the absorption maxima of the solution as sodium hydroxide concentration increases. Disruption of hydrogen bonds occurs with the relaxation of the polymer helix and subsequently, the Congo red complexes with the carbohydrate.

It has been previously shown that an ordered (e.g. helical) conformation is essential for carbohydrates such as glucan to form complexes with the dye Congo Red. Aqueous solutions of Congo Red (44μM) were prepared at various concentrations of NaOH (1 mM to 300 mM). Glucan sulfate was added to these

solutions at a final concentration of 5 mg/ml. Absorption maxima for the glucan-Congo Red solutions were determined using an LKB Ultrospec II spectrophotometer (LKB Instruments, Inc. Gaithersburg, MD). Shift in maximal absorption to shorter wavelength at elevated pH (at increased OH^- concentrations) indicates disruption of intramolecular hydrogen bonding, with subsequent transition of the helical conformation to that of a random coil, preventing the polymer from complexing with Congo Red. Inhibition of formation of the glucan-Congo Red complex results in alteration in absorption spectra of the dye in the visible wavelengths between 400 and 600 nm.

The data for glucan sulfate A (see FIG. 2A) show a shift in the absorption maxima which is not consistent with that observed for compounds which are triple helical in nature. Although applicant does not intend to be limited to this explanation, it is presently thought that the response of glucan sulfate A may suggest that the molecule is composed of both linear and triple-helical polymers.

The data for glucan sulfate B (see FIG. 2B) show a shift in absorption maxima which is consistent with that observed for compounds which are triple helical in nature. The shift in absorption maxima indicates that at least a portion of the polymers exhibit an ordered conformation.

The data for glucan phosphate (see FIG. 2C) are also shown for comparison. It too shows a shift in absorption maxima which is consistent with that observed for triple helical compounds.

5.2.1.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Carbon-13 nuclear magnetic resonance spectroscopy (^{13}C-NMR) using a Bruker 200 MHz NMR spectrometer (Bruker Instruments, Inc., Billerica, MA) was performed to determine the nature of interchain-linkages in glucan sulfate A and glucan sulfate B. The ^{13}C-NMR spectra of glucan phosphate (see FIG. 3A) and laminarin (see FIG. 3B), a linear β-1,3 linked glucopyranose standard, were also obtained for comparison.

Conditions under which the NMR spectra were obtained are as follows:

	Glucan Sulfate A	Glucan Sulfate B
Field Strength:	50 MHz	50 MHz
Relaxation Delay:	1 sec.	1 sec.
Pulse Window:	4 (15°)	6 (20°)
Number of Scans:	32030	19433

The ^{13}C-NMR spectrum obtained for glucan sulfate B (see FIG. 3C) is consistent with β-1,3 interchain linkages. The spectrum obtained for glucan sulfate A (see FIG. 3D) is not. Based on current knowledge, it was not possible at present to make definitive carbon assignments for glucan sulfate A.

5.3 USES OF SOLUBLE GLUCANS

5.3.1 PROPHYLAXIS AND/OR THERAPY OF INFECTIOUS DISEASES

Due to the potent activity of soluble glucans in stimulating the immune response and reticuloendothelial system, soluble glucans are advantageously useful in prophylactic and therapeutic applications against diseases induced by a variety of microorganisms. Because soluble glucans influence very fundamental host defense systems of the body regulating the number, functional activity and

interaction of macrophages, T and B lymphocytes, leukocytes and natural killer cells as well as their humoral and secretory components, they possess the potential for non-specifically modifying an extensive array of infectious diseases.

The soluble glucans may be used either alone or in combination with known antimicrobial agents to prevent and/or treat diseases induced by gram positive bacteria including, but not limited to: Staphylococcus aureus, Streptococcus pneumoniae, Mycobacterium tuberculosis, Haemophilus influenzae, Diplococcus pneumoniae; gram negative bacteria including, but not limited to: Escherichia coli, Bacterium enteritis, Francisella tularensis; acid-fast bacteria including, but not limited to: Mycobacterium leprae; viruses including but not limited to: Hepatitis; Herpes simplex I and II; etc.; fungi including, but not limited to: Candida albicans; Sporotrichum schenkii; and protozoal parasites including but not limited to: Leishmania donovani, Schistosoma mansoni, etc.

Additionally, the soluble glucans may be used for the prevention and/or treatment of opportunistic infections in animals and man which are immunosuppressed either as a result of congenital or acquired immunodeficiency or as a side-effect of chemotherapeutic treatment.

Soluble glucans of the present invention can be used either alone or in combination with a broad range of antimicrobial agents effective against diseases induced by bacteria, fungi, viruses and parasitic organisms. Table 5 of U.S. Patent No. 4,761,402 presents a non-exhaustive list of some of the antimirobial agents that may be used in

combination with the present soluble glucans. Table 5 is incorporated herein by reference.

Soluble glucans demonstrate a number of characteristics which make them particularly advantageous for the treatment of infections including, but not limited to the following advantages:

(1) Soluble glucans have a broad range of activity. They are effective against infections induced by fungi and viruses, and may be used effectively against infections induced by bacteria and parasitic organisms;

(2) Soluble glucans may be used additively or synergistically in combination with bioactive agents conventionally used to treat infections including but not limited to aminoglycoside antibiotics, etc.;

(3) Soluble glucans do not induce the development of resistance in causative organisms because their effects are mediated by the host;

(4) Soluble glucans have very low toxicity;

(5) Soluble glucans enhance a variety of diverse aspects of cellular and humoral immune responses of the host; and

(6) Soluble glucans stimulate hematopoiesis as evidenced by increased bone marrow proliferation.

(7) Soluble glucans may be used to prevent or reverse the development of immunosuppression in the host.

5.3.2 THERAPY OF NEOPLASMS

Due to the stimulation of macrophage phagocytic and secretory activity and increased proliferation of macrophages caused by soluble glucans, soluble glucans may advantageously be used either alone or in combination with other modalities such as surgery and chemotherapy, to treat malignant neoplastic diseases including, but not limited to adenocarcinoma, reticulum cell sarcoma, etc.

Additionally, soluble glucans directly increase the median survival time of organisms challenged with tumor cells including, but not limited to, syngeneic melanoma and syngeneic reticulum cell sarcoma, thus suggesting they decrease the rate of progression of such diseases.

5.4 ROUTES OF ADMINISTRATION

The soluble glucans of the present invention can be administered for prophylactic and/or therapeutic applications by a number of routes, including but not limited to: orally; by injection including but not limited to intravenously, intraperitoneally, subcutaneously, intramuscularly, etc.; by topical application to nasal and nasopharyngeal linings; and by inhalation via aerosolization and application to respiratory tract linings, etc.

When administered to an animal or a human, the soluble glucans may be combined with water, an aqueous solution or any physiologically acceptable pharmaceutical carrier or vehicle.

The following series of Examples are presented for purposes of illustration and not by way of limitation on the scope of the invention.

6. PREPARATION OF SOLUBLE GLUCANS

Particulate glucan was prepared from Saccharomyces cerevisiae according to the method of Di Luzio et al. (1979, Int'l J. Cancer 24: 773-779). Briefly, using a 6 l flask, 540 gm of dry yeast (Universal Foods Corp., Milwaukee, WI) was suspended in 3 l of 3% aqueous sodium hydroxide solution. The suspension was placed in boiling water bath for 4 hours, cooled overnight and the supernatant decanted. This procedure was repeated three times. The residue was then acidified with 800 ml of concentrated hydrochloric acid plus 2 l of 3% hydrochloric acid and placed in a boiling water bath for 4 hours. The suspension was allowed to stand overnight and the supernatant decanted. The residue was further digested with 3 l of 3% hydrochloric acid at 100°C for 4 hours, cooled overnight and decanted. The 3% hydrochloric acid digestion was repeated twice. The residue was then washed three times with distilled water (20°C) and twice with distilled water at 100°C. One l of ethyl alcohol was added to the residue, mixed thoroughly and allowed to stand a minimum of 24 hours for maximum extraction. The dark reddish-brown alcohol supernatant was aspirated from the residue and discarded. The alcohol extraction procedure was repeated until the alcohol supernatant was essentially colorless. The alcohol was removed by washing the residue four times with hot water; the particulate glucan preparation was then collected by centrifugation, frozen and lyophilized.

6.1 PREPARATION OF GLUCAN SULFATE

Glucan sulfate was prepared according to the present invention by solubilization and sulfation of the particulate glucan as follows:

72 gm of urea (8 M) was added to a flask containing 200 ml dimethylsulfoxide (DMSO) and stirred until dissolved. Four grams of particulate glucan were added and stirred until dissolved. The flask was heated to about 100°C and 32 ml of concentrated sulfuric acid (14M) was added. The mixture was maintained at 100°C for 6 hours by immersion in a boiling water bath. It is preferred to allow the reaction to proceed for about 6 hours.

During the heating process, a precipitate was formed which became visible after about 1.5 hours and increased in amount thereafter. After about 6 hours, the mixture was cooled and diluted with 4 l of water to resuspend the precipitate. The mixture was then filtered through coarse (1-3 μ) medium (0.65 μ) and fine (0.45 μ) sintered Millipore filters to remove the precipitate.

The resulting solution was then molecularly sieved in order to remove low molecular weight (MW) fractions including glucose, DMSO, urea and unreacted sulfuric acid.

In one series of experiments, molecular sieving was accomplished using a Millipore dialyzer/concentrator (Millipore Corp., Bedford, MA) with a 10,000 MW membrane filter. Dialysis against 100 L Milli-Q grade water was used to remove low MW compounds.

Following molecular sieving, the solution containing the glucan sulfate was concentrated, shell frozen and lyophilized. This glucan sulfate (glucan sulfate A) is stable in a lyophilized state for at least 2 years and at least for 15 months in solution maintained at 20°.

Glucan sulfate was prepared according to a second method of the present invention by

solubilization and sulfation of the particulate glucan as follows:

72 gm of urea (8 M) was dissolved in a flask containing 100 ml DMSO. Four grams of particulate glucan were added and stirred until dissolved. The flask was heated to about 100°C and a mixture of 10 ml of concentrated sulfuric acid (14 M) and 100 ml of DMSO was added. The mixture was maintained at about 100°C for 4 hours by immersion in a boiling water bath. It is preferred to allow the reaction to proceed for about 4 hours.

After about 4 hours, the mixture was cooled and diluted with 4 L water to resuspend the precipitate. The mixture was then filtered through a Millipore pre-filter (about 1-3 μ) to remove the precipitate.

The resulting solution was then molecularly sieved in order to remove low molecular weight (MW) fractions including glucose, DMSO and urea. After molecular sieving, the product was concentrated, shell frozen and lyophilized.

6.2 PREPARATION OF GLUCAN ACETATE

The following demonstrates a method for the preparation of glucan acetate. 10 ml of glacial acetic acid is added to 25 ml DMSO. A flask containing 1 g of particulate glucan and 18 g urea dissolved in 25 ml DMSO is heated and maintained at about 50-150°C. The DMSO/acetic acid mixture is then added. This procedure does not result in the formation of a precipitate. The reaction is allowed to proceed for 6 hours at 100°C. The material is cooled to stop the reaction, diluted with deionized water, and filtered to remove any remaining particulate or colloidal material. The solution is

then dialyzed and concentrated using, for example, tangential flow ultrafiltration against a 10,000 D cartridge filter. The resulting material is shell frozen and lyophilized. The yield using this procedure is approximately 12.5%.

7. IMMUNOBIOLOGICAL PROPERTIES OF GLUCAN SULFATES

In all experiments reported below, animals were maintained on Purina Laboratory Chow ad libitum in air-conditioned rooms maintained on 12 hour light/dark cycles.

7.1 MODIFICATION OF INFECTIOUS DISEASES IN EXPERIMENTAL ANIMALS BY IN VIVO ADMINISTRATION OF GLUCAN SULFATES

Previous studies have demonstrated that particulate glucan and glucan phosphate will significantly modify the course of a variety of experimentally induced infectious diseases, including, but not limited to, those of bacterial, fungal, viral and parasitic origins (see U.S. Patent Nos. 4,739,046; 4,761,402; 4,818,752 and 4,833,131; Browder et. al., 1987, Surgery 102: 206-214; review by Di Luzio, 1983, Trends in Pharmacol. Sci. 4: 344-347).

7.1.1 EFFECT OF GLUCAN SULFATES ON EXPERIMENTALLY INDUCED CANDIDIASIS

The following experiment demonstrates that administration of glucan sulfate enhanced survival in mice with experimentally induced candidiasis.

Twenty-four male ICR/HSD mice (Harlan/Sprague-Dawley, Houston, TX) were divided into two groups. Group 1, designated as the control group, received intravenous injections of dextrose (5% w/v). Group 2 received intravenous injections of glucan sulfate A (500 mg/kg) on days 10, 8, 6, 4 and 2 prior

to the induction of candidiasis and on days 3, 6, 9 and 12 following the induction of candidiasis. Candidiasis was induced by intravenous injection of 3 x 10^6 Candida albicans.

Survival of mice was monitored for 60 days following infection with the fungus. Results are illustrated in FIG. 4.

As demonstrated in FIG. 4, intravenous administration of glucan sulfate significantly ($p<0.01$) increased survival in mice with candidiasis. Glucan sulfate treated mice showed a 75% long-term survival rate. In contrast, control mice which received dextrose showed only 8% long-term survival. No further mortality was observed in either group past day 60.

7.1.2 EFFECT OF GLUCAN SULFATE ON EXPERIMENTALLY INDUCED VIRAL HEPATITIS

This experiment demonstrates that prior administration of glucan sulfate increases survival in mice with experimentally induced viral hepatitis.

Twenty C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were divided into two groups. Group 1, the control group, received intravenous injections of dextrose (5% w/v). Group 2 received intravenous injections of glucan sulfate A (50 mg/kg) on days 10, 8, 6, 4 and 2 prior to the induction of acute viral hepatitis by intraperitoneal injection of a 1:2.5 dilution of 16 complement fixing units of mouse hepatitis virus strain A-59.

Survival of mice was monitored for 14 days following infection with the virus. Results are illustrated in FIG. 5.

As demonstrated in FIG. 5, the administration of glucan sulfate significantly ($p<0.05$) increased long-term survival in mice. Glucan

sulfate treated mice showed a 60% long-term survival rate. In contrast, control mice which received dextrose showed 100% mortality. No further mortality was observed in either group after day 14.

7.2 ENHANCEMENT OF MACROPHAGE PHAGOCYTIC ACTIVITY

The following experiment demonstrates that administration of glucan sulfate significantly enhanced *in vivo* phagocytic function of macrophages.

Twelve C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were divided into two groups of 6 each. On days 0, 1, 2, 3, 4 and 5 prior to determination of phagocytic activity, Group 1, designated as the control group, received intravenous injections of dextrose (5% w/v). Group 2 received intravenous injections of glucan sulfate A (200 mg/kg).

Phagocytic function was evaluated by measuring the rate of intravascular clearance of ^{125}I-labelled reticuloendothelial test lipid emulsion. ^{125}I-labelled RE test lipid emulsion was administered intravenously (30 mg/mouse) and vascular clearance was assessed at 1, 3, 6 and 9 min. Results are presented in Table 6.

TABLE 6

EFFECT OF GLUCAN SULFATE A ON MACROPHAGE PHAGOCYTIC FUNCTION

Treatment	N	Intravascular Clearance t/2 (minutes)
Dextrose (5% w/v)	6	7.42 ± 0.66
Glucan Sulfate A	6	4.27 ± 0.51*

*p<0.001

Glucan sulfate A resulted in a significant (p<0.01) 42% increase in the intravascular clearance of RE test lipid emulsion, thus indicating that glucan sulfate A will increase *in vivo* macrophage phagocytic activity.

7.3 STIMULATION OF BONE MARROW ACTIVITY

Previous studies have demonstrated that glucan phosphate stimulates hematopoiesis (Patchen and MacVittie, 1986, J. Immunopharmacol. 8: 407-425; Patchen and MacVittie, 1982, in Macrophages and Natural Killer Cells, Normann and Sorkin, eds., Plnem Publishing Corp., New York, p. 267) as well as inducing hemapoietic activity following sub-lethal and lethal exposure to whole body ionizing radiation (Patchen et. al., 1988, Fund. Appl. Toxicol. 11: 573-574).

The following experiment demonstrates that *in vivo* administration of glucan sulfate stimulated

bone marrow activity as assessed by an in vitro assay of bone marrow proliferation.

Twenty-four male C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) (about 18 g) were intravenously injected with glucan sulfate A (250 mg/kg). Twenty-four control mice received isovolumetric dextrose (5% w/v). Bone marrow cells were harvested at 24 hours. In vitro bone marrow proliferation was assessed by the method of Williams et. al. (1987, Hepatology 7: 1296-1304).

Briefly, mice were sacrificed by ether euthanasia. Bone marrow cells were collected by aspiration of the femur with physiological saline (3 ml) using a 25 gauge needle. Bone marrow cells were centrifuged, washed (2X) and resuspended to 1 x 10^6/ml in RPMI-1640 media (Irvine Scientific, Santa Ana, CA). Cells were aliquoted into 96 well plates (1 x 10^5/ well) containing RPMI-1640 with a final volume of 300 μl. Bone marrow cells were incubated for 40 hours and pulse-labelled with ^{3}H-thymidine (1 μCi/well) for 8 hours. Following incubation, the plates were centrifuged, washed (2x) with physiological saline and 200 μl of distilled water was added to each well. The plates were freeze-thawed (3x) and 100 μl of the resulting supernatant added to 4 ml of Ecoscint (ICN Radiochemicals, Irvine, CA) and counted in an LKB Wallac Rackbeta scintillation counter (LKB Instruments, Gaithersburg, MD).

The effect of glucan sulfate A on murine bone marrow proliferation is shown in Table 7.

TABLE 7

EFFECT OF GLUCAN SULFATE A ON BONE MARROW PROLIFERATION

Treatment	^{3}H-Thymidine Uptake (counts per minute)
Dextrose (5% w/v)	1666.7 ± 139.3
Glucan Sulfate A	2440.4 ± 113.6*

*p<0.041

As demonstrated by Table 7, cells obtained from mice treated with a single <u>in vivo</u> administration of glucan sulfate A showed increased <u>in vitro</u> bone marrow proliferation as reflected by the significant 46% ($p<0.04$) increase in ^{3}H-thymidine uptake.

7.4 ANTI-TUMOR ACTIVITY

Previous studies have demonstrated that both particulate glucan (Sherwood et. al., 1987, J. Leukocyte Biol. 42: 69-75; Williams et. al., 1985, Hepatology 5: 198-206) and glucan phosphate (Sherwood. et. al., 1988, J. Biol. Resp. Modif. 7: 185-198) exert significant antitumor activity in various murine tumor models. The following experiments demonstrate the anti-tumor activity of the soluble glucan sulfate A.

7.4.1 MODIFICATION OF SYNGENEIC MELANOMA

The following experiment demonstrates that administration of glucan sulfate A increased median survival time for mice challenged with syngeneic melanoma.

Twenty C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were divided into 2 groups of 10 each. All mice received subcutaneous injection of 5.0×10^5 syngeneic melanoma B16 cells. Thereafter, Group 1, designated as the control group, received dextrose (5% w/v) twice weekly; Group 2 received glucan sulfate A (250 mg/kg) twice weekly. All injections were given intravenously or intraperitoneally.

As demonstrated in Table 8, significantly increased median survival times ($p<0.001$) were observed in mice treated with glucan sulfate A. However, ultimate survival (i.e., the time to 100% mortality) was not significantly altered in that population receiving glucan sulfate A.

TABLE 8

EFFECT OF GLUCAN SULFATE A ON SURVIVAL OF MICE WITH SYNGENEIC MELANOMA B16

Treatment	N	Median Survival Time (days)
Dextrose 5% (w/v)	10	25.5
Glucan Sulfate A	10	33.0*

*p<0.001

7.4.2 MODIFICATION OF RETICULUM CELL SARCOMA

The following experiment demonstrates that administration of glucan sulfate A increased median survival time for mice challenged with syngeneic reticulum cell sarcoma.

Twenty-nine C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were subcutaneously

injected with 5.0 x 10^5 syngeneic reticulum cell sarcoma M5076 cells in the scruff of the neck on day 0. On day 0 and at 3 day intervals thereafter, Group 1, consisting of 17 mice designated as the control group, received dextrose (5% w/v) intravenously; Group 2, consisting of 12 mice, received glucan sulfate A (250 mg/kg).

As demonstrated in Table 9, significantly increased median survival times ($p<0.001$) were observed in mice treated with glucan sulfate A. However, ultimate survival (i.e., the time to 100% mortality) was not significantly altered in the population receiving glucan sulfate A.

TABLE 9

EFFECT OF GLUCAN SULFATE A ON SURVIVAL OF MICE WITH SYNGENEIC MELANOMA B16

Treatment	N	Median Survival Time (days)
Dextrose 5% (w/v)	17	37.5
Glucan Sulfate A	12	43.5*

*p<0.001

WHAT IS CLAIMED IS:

1. A soluble glucan, comprising a poly-glucopyranose chain having a polar charged group selected from the group consisting of sulfate, nitrate and acetate groups, characterized by:

(a) the capability of dissolving in water or an aqueous solution;

(b) being non-toxic; and

(c) the capability of exerting a pronounced immunobiological response when administered in vivo.

2. The soluble glucan according to claim 1, having a sulfate group.

3. The soluble glucan according to claim 2, further characterized by aqueous solubility of about 400 mg/ml.

4. The soluble glucan according to claim 2, further characterized by aqueous solubility of about 50 mg/ml.

5. The soluble glucan according to claim 1 which is obtained from a microbial source.

6. The soluble glucan according to claim 5, in which the microbial source is Saccharomyces cerevisiae.

7. The soluble glucan according to claim 1, in which the poly-glucopyranose chain is further characterized by having an average molecular weight substantially within the range of about 10,000 to about 100,000 daltons.

8. A method for preparing a non-toxic, soluble glucan having a polar charged group selected from sulfate, nitrate and acetate groups and capable of exerting a profound immunobiological response when administered in vivo, comprising:

(a) solubilizing a particulate glucan in a highly polar solvent which contains a strong chaotiopic agent;

(b) reacting the resultant solubilized glucan with a non-phosphorous-containing hydrolytic acid selected from sulfuric, nitric and acetic acids to form a soluble glucan; and

(c) recovering the resultant soluble glucan having a charged, polar group from the reaction mixture.

9. The method according to claim 8, in which the strong chaotropic agent comprises urea.

10. The method according to claim 8, in which the highly polar solvent is dimethylsulfoxide.

11. The method according to claim 8, in which the particulate glucan is solubilized by a method comprising the steps of:

(a) suspending a particulate glucan in a highly polar solvent which contains a strong chaotropic agent to form a mixture;

(b) heating the mixture to about 50-150°C;

(c) adding a non-phosphorous-containing hydrolytic acid selected from sulfuric, nitric and acetic acids to the mixture; and

(d) allowing the mixture to react for about 1-12 hours at 50-150°C.

12. The method according to claim 8, in which the hydrolytic acid in step (b) is sulfuric acid.

13. The method according to claim 8, in which step (b) further comprises reacting the solubilized glucan with the non-phosphorous containing hydrolytic acid in the presence of a highly polar solvent to form a soluble glucan.

14. A method for treating an infection in animals or humans, comprising administering to an animal or a human affected with the infection, a therapeutically effective amount of a soluble glucan which comprises a poly-glucopyranose chain having a polar charged group selected from the group consisting of sulfate, nitrate and acetate groups, characterized by:

(a) the capability of dissolving in water or an aqueous solution;

(b) being non-toxic; and

(c) the capability of exerting a pronounced immunobiological response when administered in vivo.

15. The method acording to claim 14, in which the infection is induced by a bacterium, a virus, a fungus or a parasite.

16. The method according to claim 14, in which the bacterium is selected from the group consisting of Staphylococcus aureus, Streptococcus pneumoniae, Mycobacterium tuberculosis, Hemophilus influenzae, Escherichia coli, Bacterium enteritis, Francisella tularensis, and Mycobacterium leprae.

17. A method for treating a neoplastic disease in animals or humans, comprising administering to an animal or a human affected with the neoplastic disease, a therapeutically effective amount of a soluble glucan which comprises a poly-glucopyranose chain having a polar charged group selected from the group consisting of sulfate, nitrate and acetate groups, characterized by:

(a) the capability of dissolving in water or an aqueous solution;

(b) being non-toxic; and

(c) the capability of exerting a pronounced immunobiological response when administered in vivo.

18. A pharmaceutical composition for the prevention or treatment of an infection in animals or humans, comprising a therapeutically, or prophylactically effective amount of a soluble glucan composition which comprises:

(a) a poly-glucopyranose chain having a polar charged group selected from the group consistin of sulfate, nitrate and acetate groups, characterized by:

(1) the capability of dissolving in water or an aqueous solution;

(2) being non-toxic; and

(3) the capability of exerting a pronounced immunobiological response when administered in vivo, and

(b) a pharmaceutically acceptable carrier.

19. The composition acording to claim 19, further comprising an antibiotic agent, effective against the infection.

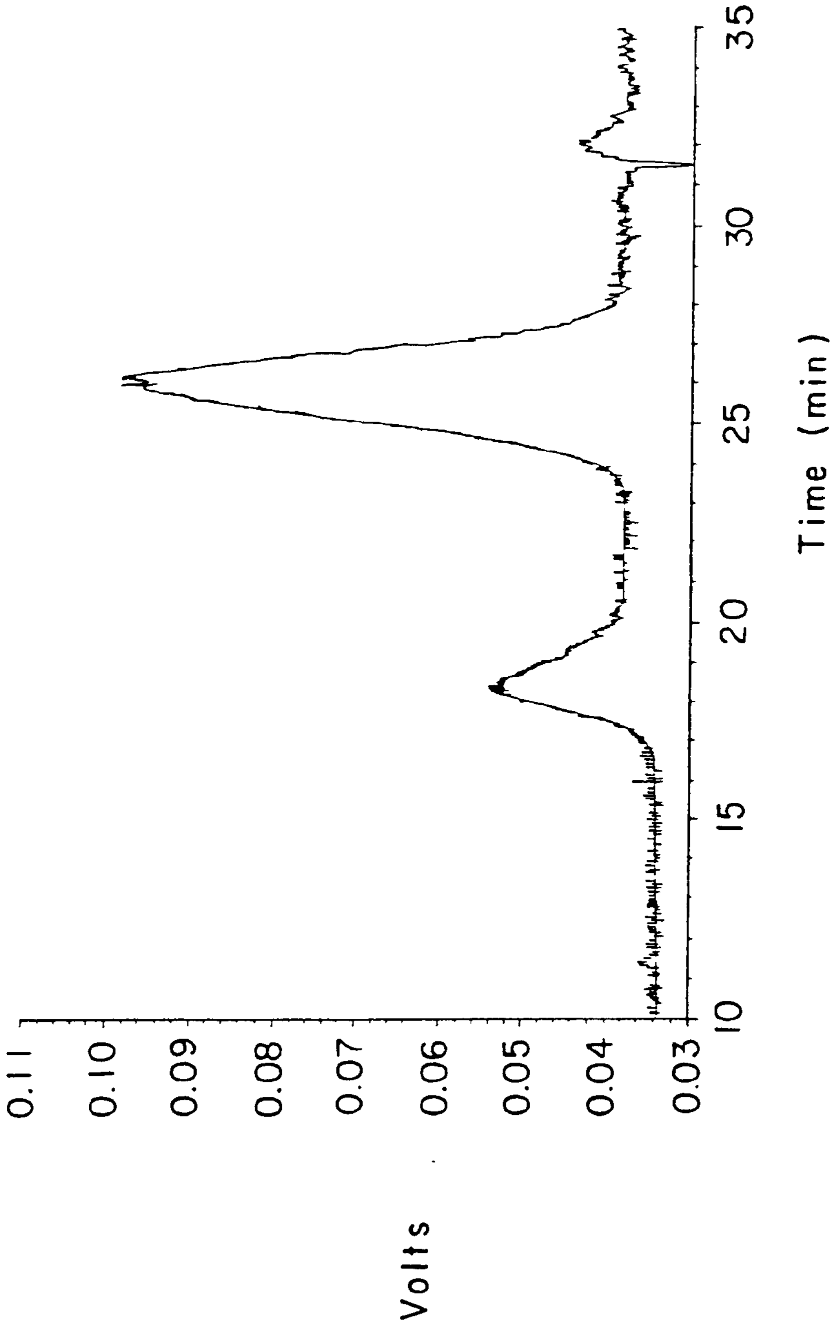

Volts
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
10
15
20
25
30
35
Time (min)

FIG. 1A

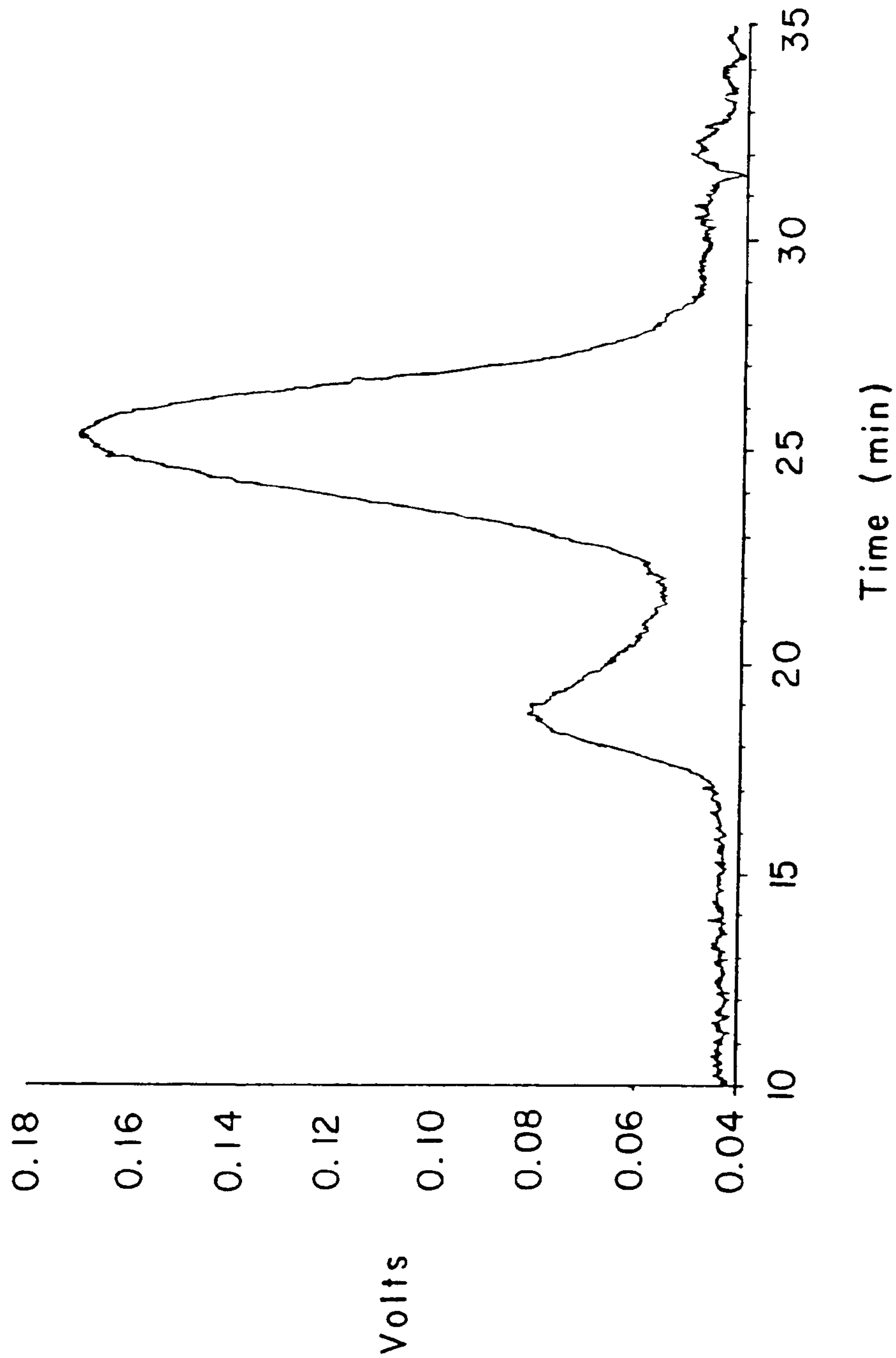


FIG. 1B

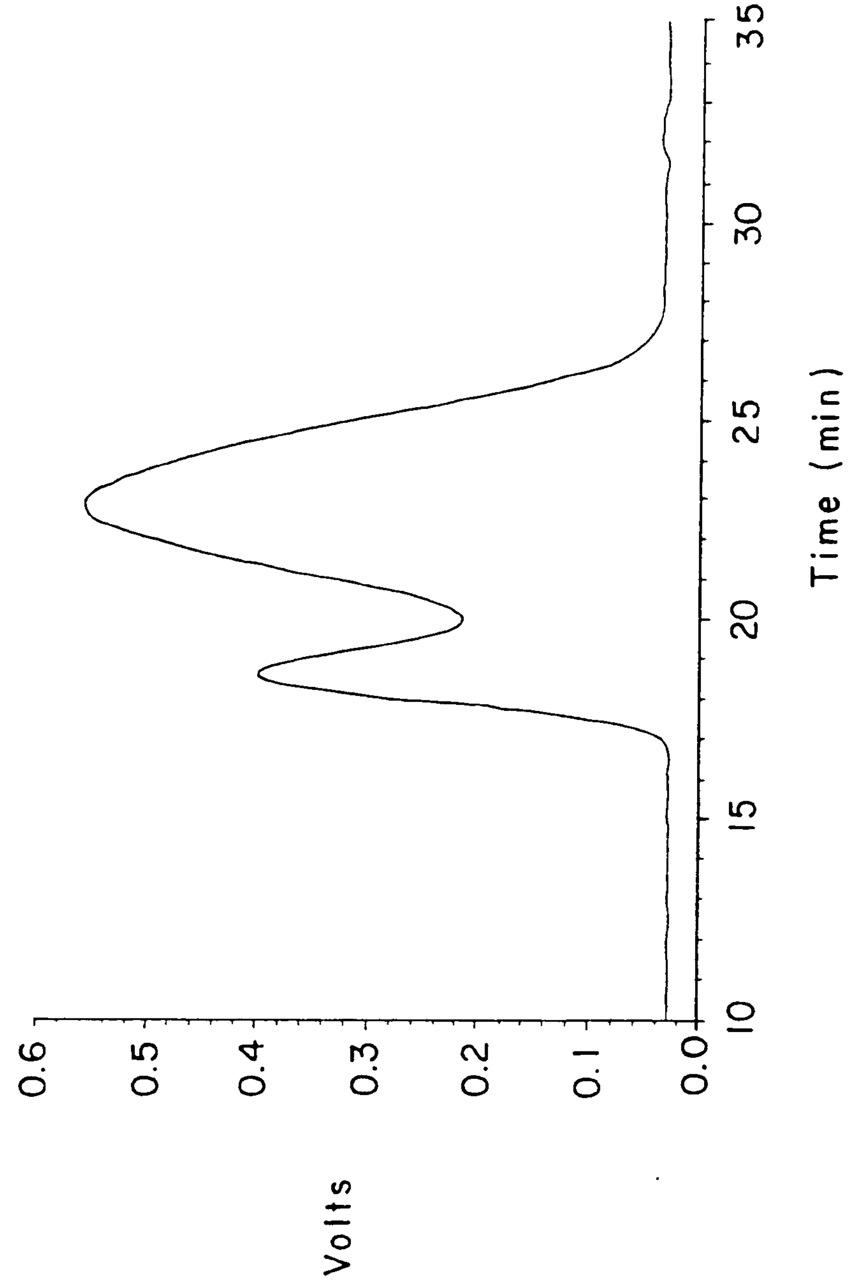


FIG. IC

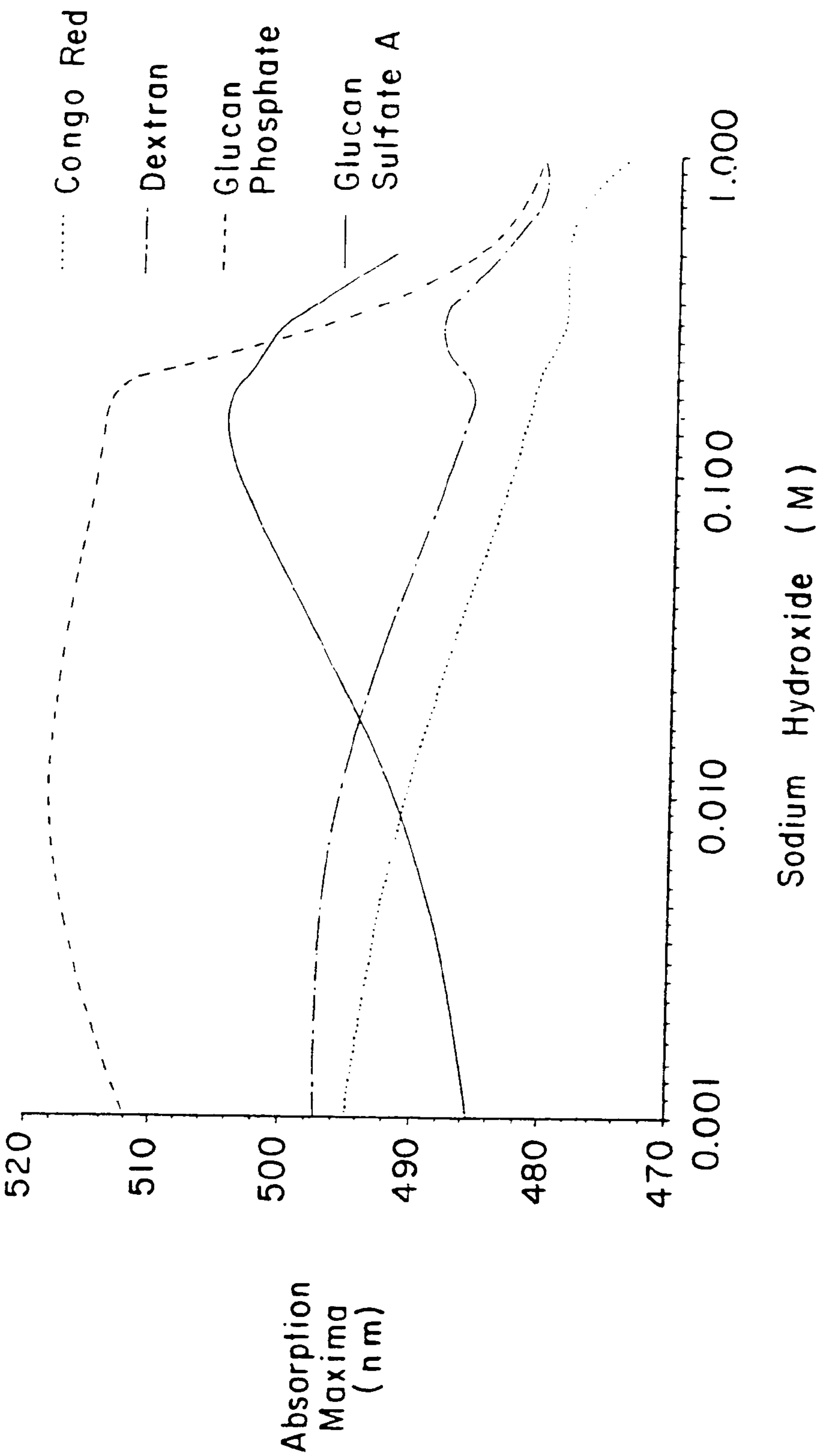
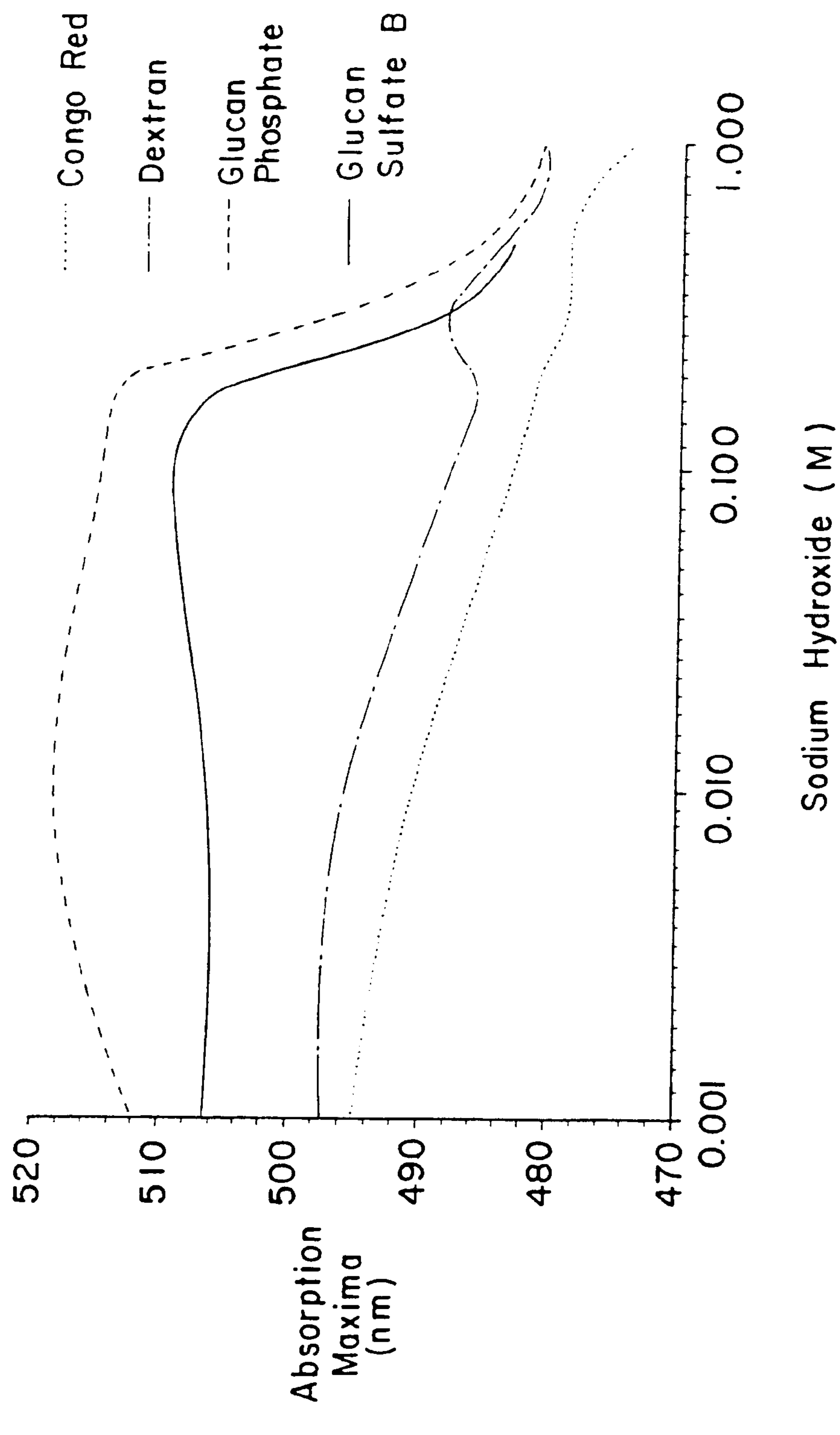

Congo Red
Dextran
Glucan Phosphate
Glucan Sulfate A
Absorption Maxima (nm)
520
510
500
490
480
470
0.001
0.010
0.100
1.000
Sodium Hydroxide (M)

FIG. 2A



Congo Red
Dextran
Glucan Phosphate
Glucan Sulfate B
Absorption Maxima (nm)
520
510
500
490
480
470
0.001
0.010
0.100
1.000
Sodium Hydroxide (M)

FIG. 2B

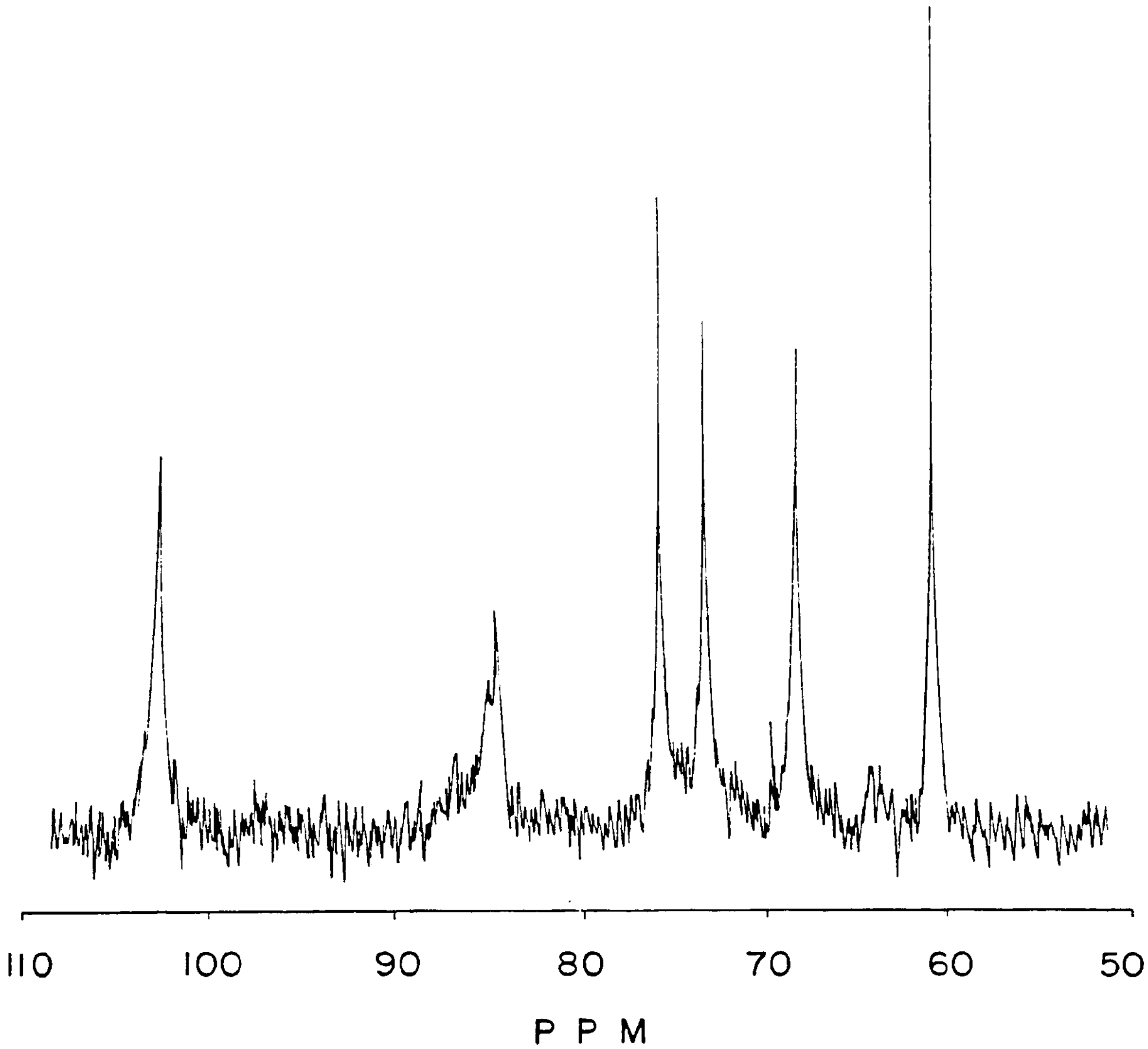


FIG. 3A

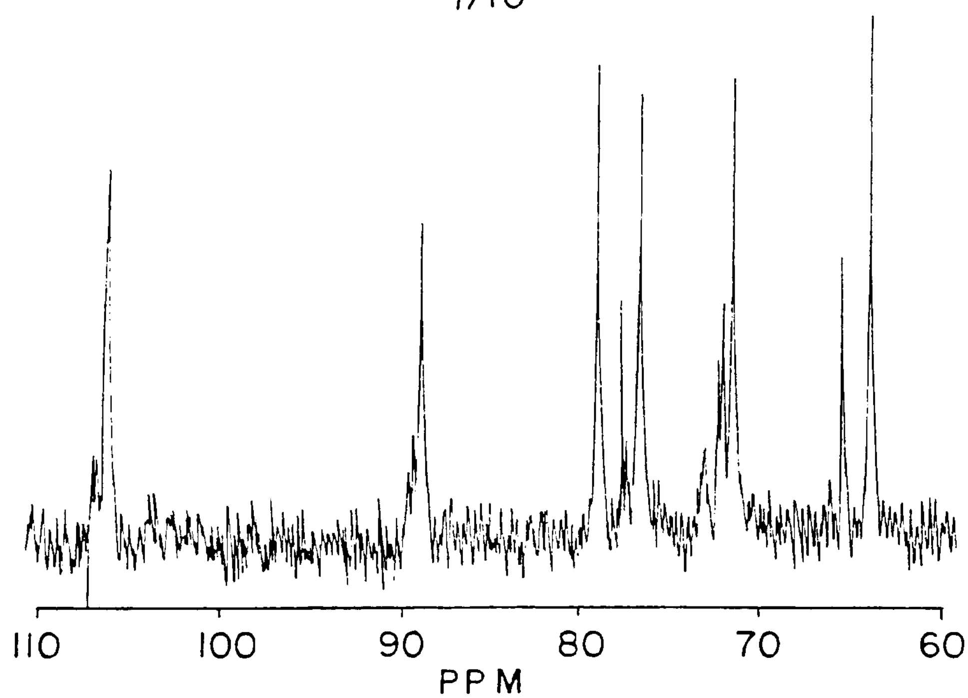


FIG. 3B

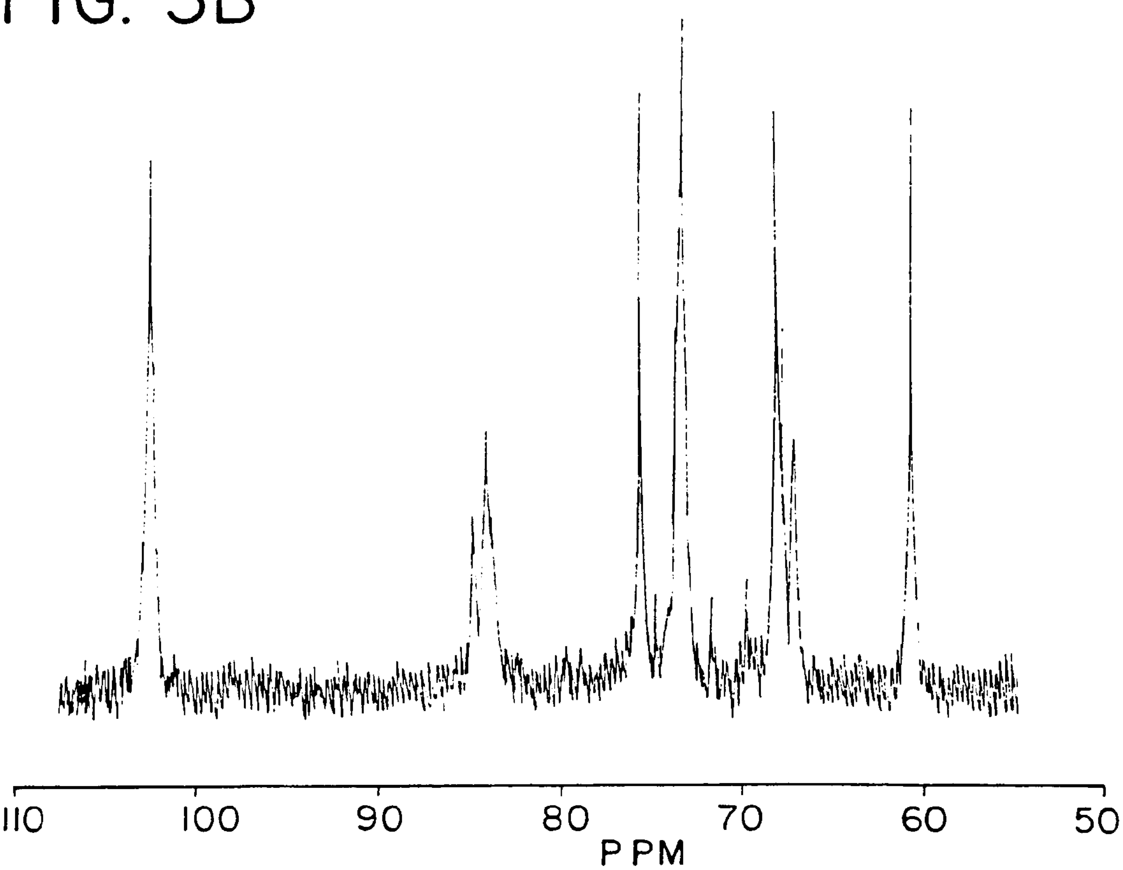


FIG. 3C

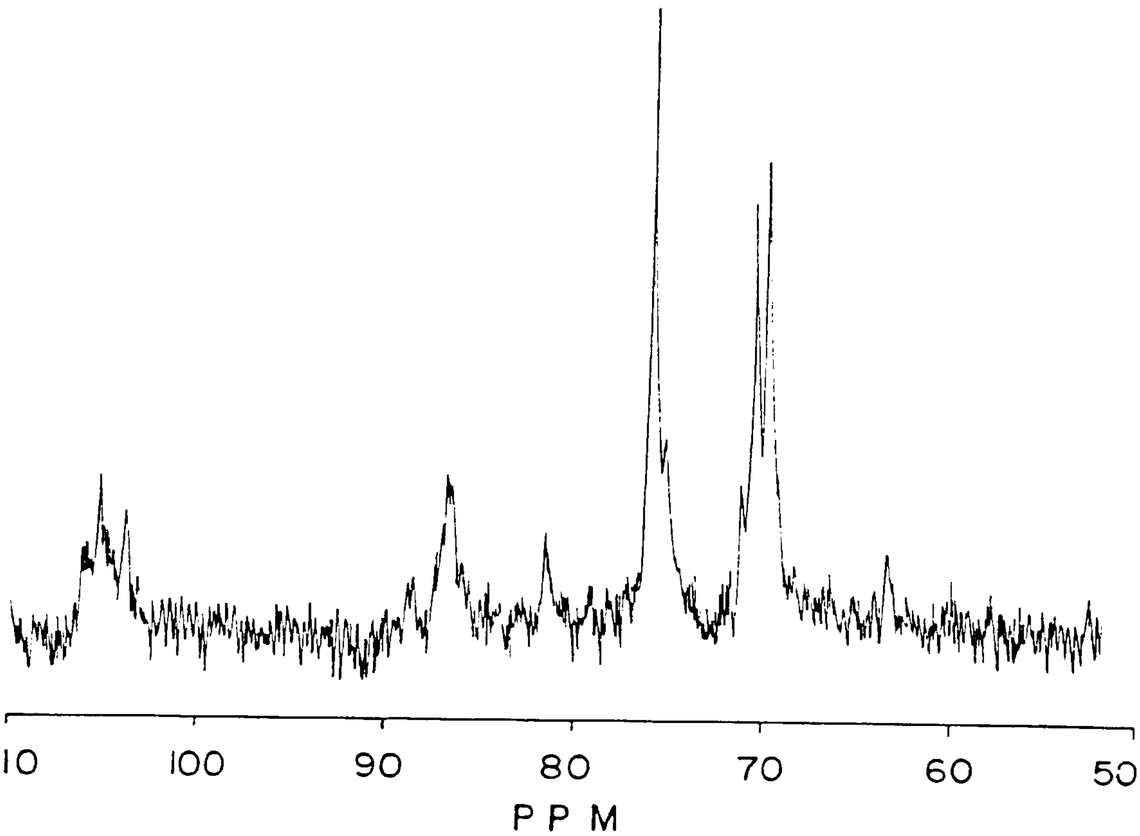
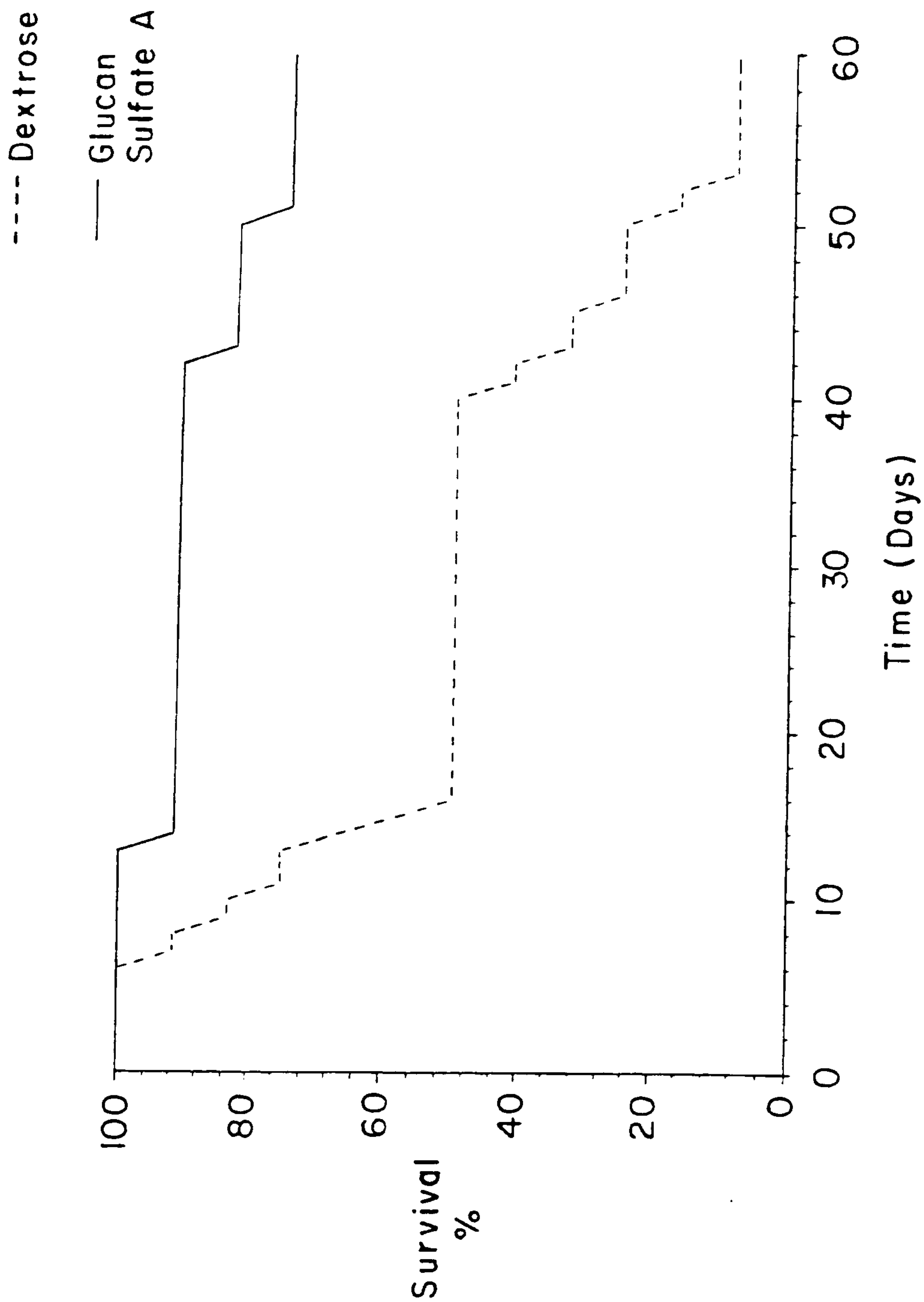


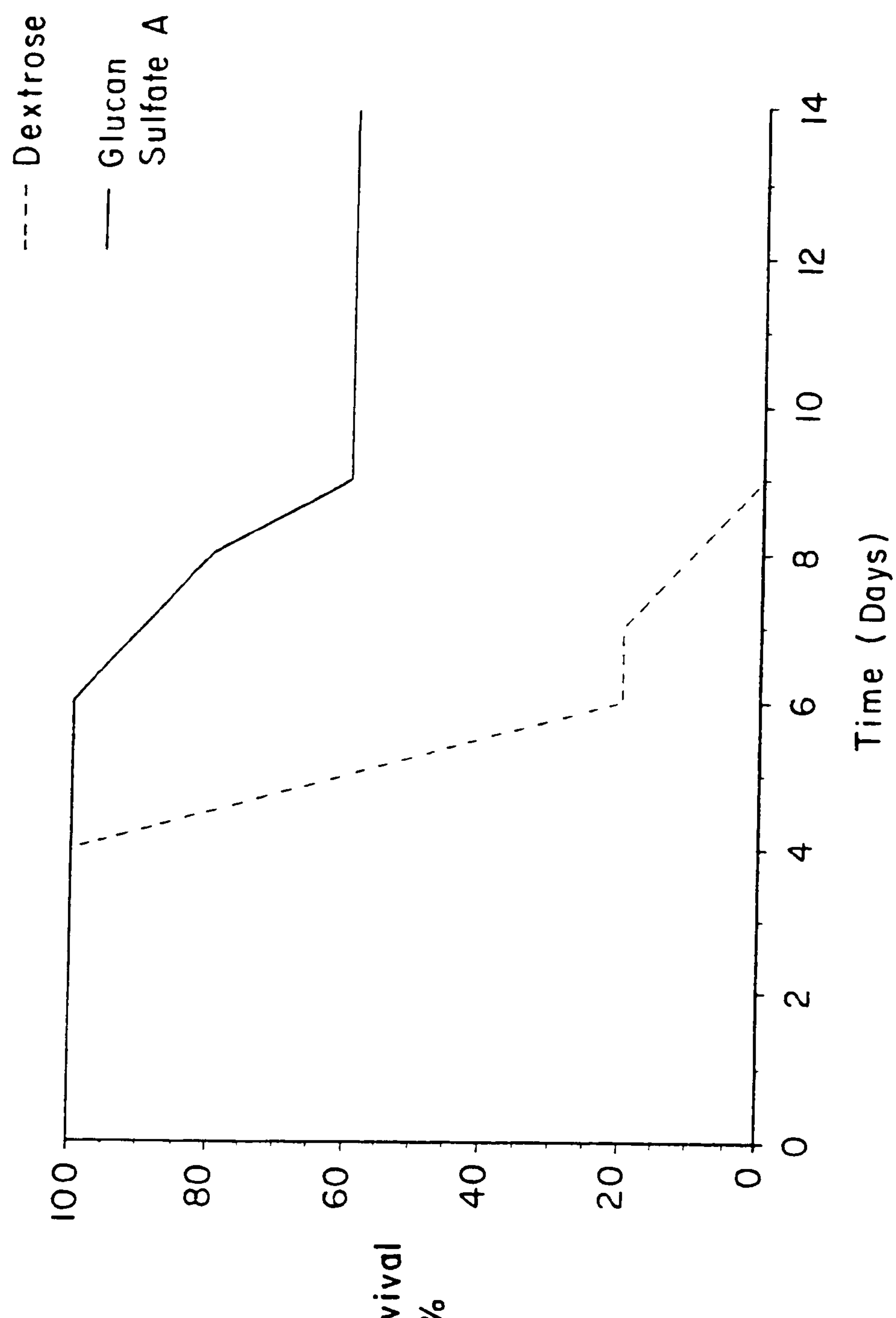
FIG. 3D

SUBSTITUTE SHEET



Dextrose
Glucan Sulfate A
Survival %
100
80
60
40
20
0
10
20
30
40
50
60
Time (Days)

FIG. 4



Dextrose
Glucan Sulfate A
Survival %
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time (Days)

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00866

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C08B 37/16, 37/00; A61K 9/06, 31/715
US CL : 514/54,56; 536/1.1, 114, 117, 53

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	514/54,56; 536/1.1, 114, 117, 53

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US,A, 4,705,780 (MASSOT et al) 10 November 1987, see entire document.	1-19
Y	US,A, 4,965,347 (Misaki et al) 23 October 1990, see entire document.	1-19
Y	US,A, 4,795,745 (Larm et al) 03 January 1989, see entire document.	1-19
A,&	US,A, 4,739,046 (Di Luzio) 19 April 1988.	1-19
A	US,A, 3,985,727 (Daniels) 12 October 1976.	1-19
A	US,A, 4,761,402 (Williams et al) 02 August 1988.	1-19

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
19 MAY 1992	04 JUN 19[illegible]
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	LOUISE N. LEARY

Form PCT/ISA/210 (second sheet)(May 1986) B